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Research Article

Effect of solvent type on phytochemical properties of burdock (*Arctium lappa*) extract and their effect on some pathogenic bacteria strains in rainbow trout, *Oncorhynchus mykiss*

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Abstract

The extraction method and particularly the type of solvents used in the procedure are one of the most crucial steps for isolating antimicrobial compounds and extracts that potentially affect various microorganisms. This study aimed to evaluate the effects of water, ethanol (100%), methanol (100%), ethanol-water (50:50), and methanol-water (50:50) burdock (*Arctiumlappa*) root extracts (BE) on total phenol content and polyphenols as well as the antioxidant activity. In the next step, the solvent with the highest efficiency to have phenolic compounds were selected and employed to investigate the antibacterial effects of BE on the pathogenic bacterial strains (*i.e. Yersinia ruckeri*, *Lactococcus garvieae*, *Pseudomonas putida* and *Aeromonas hydrophila*) in rainbow trout (*Oncorhynchus mykiss*) by using disc diffusion test and tube dilution techniques. Maximum mean total phenolic content and antioxidant capacity were detected in the water-methanol mixture. Additionally, High-performance liquid chromatography(HPLC) analysis revealed that both hesperidin and trans-ferulic acid compounds existed in all solvents. The antimicrobial activity of water-methanol BE was varied based on the type of microorganism. In this context, *Y. ruckeri* as a gram negative bacterium was found to be the most susceptible one compared to other pathogens. According to our findings, water-methanol solvent was more efficient to isolate phenolic compounds and exert antioxidant capacity, as the level of phenolic compounds in the extract was increased. Moreover, *Y. ruckeri* was recognized as the most sensitive pathogen in all tests.

Keywords: Polar and nonpolar solvents, *Arctium lappa* root extract, Antibacterial, Rainbow trout, Phenol

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Introduction

Burdock plant (*Arctium lappa*) is an herbaceous biennial plant belonging to the Asteraceae family. Having a thick branched stem, this plant grows wild in the humid and moderate plains and regions in Europe and Asia (Predes *et al.*, 2009). Burdock has antifungal, antiviral, anti-inflammatory, and detoxification properties (Ferracane *et al*., 2010). Burdock root contains chemical compounds including inulin, volatile oils, tannins, resin, sugar, iron, calcium, quercetin, arctigenin, and vitamin C. Furthermore, this flavonoid-containing plant has also remarkable antioxidant activities (Ferracane *et al*., 2010). Burdock contains different kinds of lignan compounds, notably lapazole C, F, A,matairesinol, arctigenin, arctinin, arctignan E. Arctin as a lignin has been found in the root and it can significantly reduce the elevated level of Malondialdehyde (MDA) as a marker of oxidative stress (Chan *et al*., 2011).

Due to their hydroxyl groups, phenols have the ability for scavenging free radicals, they are among the very important ingredients in plants and may directly contribute to the oxidative effect of the plants (Gulcin *et al.*, 2002). Effects of phenolic compounds may be included to their capacity for scavenging free radicals, metal-chelating properties, gene expression regulation and also their co-antioxidant activity (Neergheen *et al*., 2006). Apparently, there is a close relationship between antioxidant activity and total phenolic contents (Davarynejad *et al*., 2012). A large number of studies have been conducted on antioxidant compounds and several synthetic antioxidants are also available which using them has been limited due to their toxicity. Therefore, finding natural antioxidants remarkably derived from plants and using them in food and drug industries are a pressing need. Employing these plant-derived natural compounds has some advantages including extensive biological effects, especially in controlled concentrations (Milos *et al*., 2000). It should be noted that a single method isn't efficiently enough to survey antioxidant activity, but a combination of several procedures is an acceptable approach to assess antioxidant activity of various extracts (Kulisic *et al.*, 2004). In general, there are water and organic solvents including ethanol, acetone and diethyl-ether to extract polyphenols from the plants (Sun and Ho, 2005; Turkmen *et al.*, 2006; Hayouni *et al.*, 2007). There are obvious differences between phenolic contents in different extracts that may have been caused by variations in sample preparation approaches, the method used in the procedure, the duration of extraction process, and the physicochemical properties of the used solvents. By Rezaei *et al*. (2015) the effects of different groups of solvents and total phenolic content of Saqez (*Pistacia atlantica*) shell and the efficiency of their extraction were investigated and there was a significant difference that was attributed to the polarity, viscosity and unique vapor pressure of each

solvent . Thus, introducing a solvent with a defined concentration and maximum efficacy to isolate phenolic and antioxidant compounds from a certain plant is not an easy task.

Rainbow trout is the most important species among cold-water fish species in most parts of the world. Due to the increasing desire to breed this species, there are growing reports of arising different diseases such as bacterial diseases. *Lactococcus garvieae*, *Yersinia ruckeri*, *Aeromonas hydrophila* and *Pseudomonas putida* are the important underlying causes of septicemia and high mortality in fish farms especially in rainbow trout farms (Zorriehzahra *et al*., 2017). Since different extracts display varied levels of antimicrobial activity against various strains of bacteria, it seems that this antibacterial activity is derived from different chemical properties of particular ingredients in the plants. Isolation efficacy of these substances depends upon the type of solvent used for dissolving the solute. Hence, taking different approaches in extraction and employing various kinds of solvents may lead to more efficient extraction of biological substances with antibacterial properties, giving rise to developing new products with enhanced antibacterial activities (Harborne, 1998).

The aim of the current study is to investigate the effects of solvent type on the total phenolic compounds content, polyphenols, and antioxidant activity of Burdock root extract and finally evaluate the impacts of the extract with the most efficient solvent (regarding total phenolic content and antioxidant activity) against several pathogenic bacteria (gram-positive and gram-negative) in rainbow trout aquaculture industry.

Materials and methods

Preparing burdock plant and extraction procedure

Burdock plant was collected in May 2016 from Sepidan region (Fars Province). The plant then was taxonomically identified and confirmed by Herbarium and Plant Systematics Laboratory in the Faculty of Basic Sciences of Shiraz University. Protecting from exposure to light and humidity, roots were air-dried at room temperature until a constant weight was obtained and then were grinded by an electric grain mill grinder.

To prepare aqueous, methanol, ethanol-water and, ethanol-water extracts, 100g of powdered roots was added to an erlenmeyer flask containing 500 mL of methanol (100%) or ethanol(100%) or distilled water or methanol-water mixture (1:1) or ethanol-water mixture(1:1). The mixture was mixed gently on a magnetic stirrer at room temperature for 72 hours till extraction was completely accomplished.

Then to obtain primary extracts, the mixture of solvent and root powder was filtered by whatman filter paper. Supernatant was centrifuged at 3000 rpm for 10 minutes and was passed through a 0.45-µm pore size filter. To remove microbial contaminations

single-use syringe filters were employed and ultimately primary extracts were concentrated in a vacuum distillation apparatus. Concentrated extracts were collected in aluminum sterile containers and stored at 4°C until the later use (Ahmad and Beg, 2001).

Quantitiation of total phenolic content

Total phenolic content was measured in terms of the data obtained from folin-ciocalteu colorimetric method. Gallic acid was employed as the reference standard (Marinova *etal*., 2005). Then 1.8 mL of distilled water and 0.2 mLof diluted folin-ciocalteu reagent (1:15 v/v) were added to 0.2 mL of each extract or reference standard (0-100 mg/mL gallic acid in distilled water). After 5 minutes, 3 mL of sodium bicarbonate solution (7%) was added and the volume was brought to 5 mL with distilled water. The samples were incubated at room temperature for 90 minutes and then the absorbance was measured at 765 nm and total phenolic content was calculated as mg GA g^{-1} DW with regard to the calibration curve.

Investigation of antioxidant activity by DPPH (1, 1diphenyl-2-picryl hydrazyl)

In this method DPPH, a lipophilic stable radical compound with an absorption maximum at 517 nm was used. Ability to donate hydrogen atom or electron was evaluated by various chemical compounds and extracts according to their ability to change purple DPPH into a colorless form. To assess the level of antioxidant properties of this plant, 35-50 µL Burdock root extract dissolved in different solvents were added to 1 mL absolute ethanol. Then 1 mL DPPH solution (0.004%) was added in dark and after 30 minutes absorbance was read by CECILL9900 spectrophotometer (made in Britain). Finally, by using the following formula, the ability of DPPH to capture free radicals was calculated (Akowuah *et al*., 2005):

$$I\ \% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

In this formula A_{blank}, A_{sample} and I% represent the optical density of the negative control (without extract), the optical density of the extracts and the percentage of DPPH free radical scavenging activity, respectively. Using the linear chart, IC50 value was calculated and percentage of inhibition was determined in mg/mL.

Quantification of polyphenolic compounds using HPLC (High-performance liquid chromatography)

An Agilent HPLC system was used for this assay. Flow rate was 0.5 mL/min and the solvents used in the mobile phase include acetonitrile, water and acetic acid (2%). C18 reversed phase was employed as the filling material. Column height and particle size were 25 cm and 5µm, respectively. Chromatograms were acquired at 320 nm by the detector and the injection volume was 20 µL. All the steps were performed at room temperature (25°C) and Chrom Gate software was used to analyze data. 0.1 mg of each standard

was dissolved in HPLC grade methanol and 0.005, 0.02, 0.04, 0.06 and 0.1 mg/mL concentrations were prepared and were loaded along with other samples in triplicates. In order to perform quantitative analysis, the resultant chromatograms were compared with standards and the concentrations of these compounds were calculated on a dry-weight basis (µg/g) (Materska and Perucka, 2005).

Preparation of bacteria

In the present study, four bacterial strains notably *Lactococcus garvieae* (ATCC 49156), *Aeromonas hydrophila* (ATCC7966), *Yersinia ruckeri* (entrobacteria) and *Pseudomonas putida* (KT2440) were isolated from rainbow trout (*Oncorhynchusmykiss*) in farms of Fars Province and their genus and species were validated by biochemical and molecular tests (Austin and Austin, 2007). Bacteria were initially cultivated in liquid medium (Tryptic Soy Broth or briefly TSB) and after 48 hours of exponential growth, were centrifuged at 3000 rmp for 10 minutes. The pellet was washed thrice with physiological serum and then was resuspended and a bacterial suspension was prepared. Turbidity was measured at 530 nm by a spectrophotometer and the suspensions were diluted until their turbidity was adjusted to the turbidity of a 0.5 McFarland standard. The suspension must contain 1.5×0^{8} CFU/mL(Valero and Salmerón, 2003).

Determining antibacterial activity of burdock extract using disc diffusion method

After determining the most efficient solvent to prepare the extract, antibacterial activity of this plant against gram-positive and gram-negative bacteria was investigated. We used the sterile blank disks manufactured by Padtan-Teb Company (Tehran, Iran). Blank disks were put into the tubes containing 50, 100, 400, 200 and 600 mg/mL extract and after 50-30 minutes (after complete absorption of extracts to the disk), the disks were completely dried at 45°C. Then 100µL of bacterial suspension was prepared with reference to the 0.5 McFarland standards and uniformLy distributed over the entire surface of the Mueller Hinton agar plates. The disks were placed on the surface of agar at a certain distance from each other and also from the edge of the plate and were fixed with gentle pressure. Plates were incubated for 24 hours at 37°C and then the diameter of the bacterial growth inhibition halo was measured precisely by a ruler (in mm). Each measurement was repeated three times. In all steps standard tetracycline disks (30µg/disk) manufactured by Padtan-Teb Ltd. (Tehran, Iran) were employed (Bauer *et al*., 1966).

Measuring minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Taking tube dilution method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration

(MBC) of burdock extract were determined. To determine MIC we used a series of 11 sterile test tubes. 10 out of 11 test tubes were used to test various dilutions and one as control. After culturing, all test tubes were incubated at 37°C for 24 hours and the level of turbidity in each tube was evaluated. In comparison with the control, the concentration of the first clear tube was regarded as MIC value. This test was performed in triplicate for each microorganism (Benger *etal*., 2004).

To determine minimum bactericidal concentration (MBC) of the extract, 1 mL of the concentration with no turbidity was cultured over the entire surface of the Mueller Hinton agar plates by using the streaking method. Plates were incubated at 37°C for 24 hours and then the lowest concentration in which no bacteria growth was detected was regarded as minimum bactericidal concentration (MBC) value (Espinel-Ingroff *et al*., 2002).

Statistical analysis

All statistical analysis was performed using SPSS v.19.0. One-way analysis of variance (ANOVA) was used to compare the means and Duncan's test to investigate the difference between the means at significance level of 0.05.

Results

The means of total phenolic content in extracts dissolved in different solvents have been compared and illustrated in Figure 1. According to our results, the greatest total phenolic content was detected in the aqueous-methanolic extract at 417±13.9 mg GA g^{-1} DW and the lowest was found in aqueous extract at 205.1±10.18 mg GA g^{-1} DW. Total phenolic content in methanolic extract, ethanolic extract and aqueous-ethanollic extract was higher than phenolic content of aqueous extract. However the difference was not significant (p>0.05).

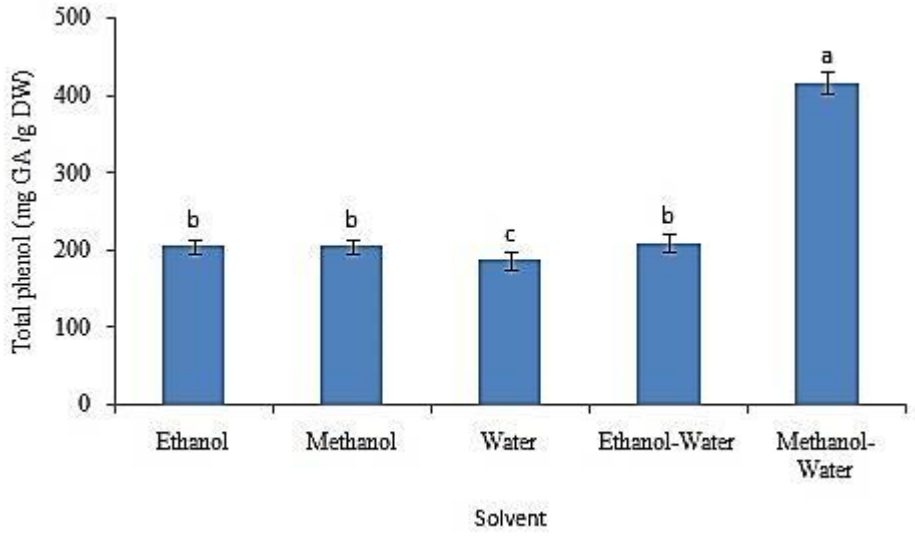

Figure 1: comparing total phenolic content of burdock root extracts dissolved in various solvents.

Unlike letters represent a significant difference between different solvents (p<0.05). The data obtained from DPPH test to measure antioxidant activity have been displayed in Figure 2. Our findings demonstrated that aqueous-methanolic extract showed the minimum IC50 of 540 and then ethanolic extract and methanolic extract were the most effective ones, respectively. Nonetheless the latter extracts showed no significant difference (p>0.05). Among them, aqueous extract represented the least antioxidant activity (IC50=590) and compared with other extracts, the difference was significant (p<0.05).Unlike letters indicate a significant difference between various solvents (p<0.05).

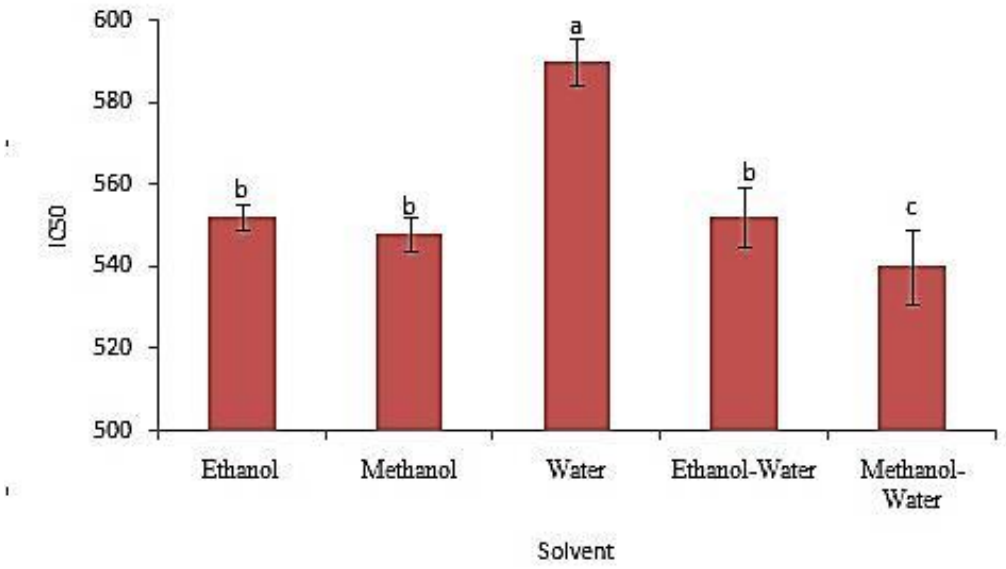

Figure 2: comparing antioxidant capacity of burdock root extracts dissolved in various solvents.

Correlation between phenolic compounds and antioxidant activity

Relationship between phenolic content and antioxidant capacity has been shown in Table1. An inverse correlation between phenolic content and IC50 of the extracts in antioxidant activity evaluations was detected (97%), indicating an increase in antioxidant activity by increasing phenolic compounds content.

Table 1: Results of correlation analysis between phenolic compounds and antioxidant activity.

Test type	Total phenol	IC50
Total phenol	1	0.975*
IC50	-	1

*Correlation is significant at the 0.05 level.

Comparing polyphenolic compounds in burdock root extracts dissolved in various solvents

HPLC analysis revealed that both hesperidin and trans-ferulic acid were found in all solvents, vanilin in all solvents except aqueous-ethanol solvent, rosmarinic acid in all solvents except ethanol and methanol, quercetin and hesperetin restrictively in aqueous solvent and chloregenic acid in ethanol and aqueous-ethanol. Other compounds were detected in none of the solvents (Table 2).

Table 2: polyphenol compounds (mg/mL) in burdock root extracts dissolved in various solvents.

Polyphenol compound (mg/L)	Water	Ethanol-Water	Methanol-Water	Methanol	Ethanol
Chloregenic acid	-	-	1.35	-	1.73
Trans- ferulic acid	15.56	21.36	16.13	32.02	31.72
Hesperidin	104.66	98.99	85.56	90.71	42.60
Vanilin	1.08	1.51	-	4.53	2.64
Quercetin	3.57	-	-	-	-
Hesperetin	6.79	-	-	-	-
Rosmarinic acid	27.90	30.10	28.80	-	-

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

With reference to the results of antioxidant activity tests and total phenolic content, burdock root extracts dissolved in aqueous-methanol solvent was the most efficient one to survey antibacterial activity. Minimum inhibitory concentration (MIC) values of this extract have been summarized in Table 3. According to our findings, MIC value of methanol-ethanol extract to inhibit *Y. ruckeri* growth was 1.25 mg/mL. MIC values of methanol-aqueous against *Y. ruckeri* , *A. hydrophila* and *P. putida* werefound to be1.25, 2.5 and 2.5(mg/mL), respectively. The results of minimum bactericidal concentration tests on burdock methanol extract have been represented in Table 3. According to our findings, MBC values of aqueous-methanol extract on *Y. ruckeri* , *A. hydrophila* and *P.putida* were 5, 10 and 10 (mg/mL), respectively (Table 4).

Table 3: Quantifying minimum inhibitory concentrations (MIC) of aqueous-methanol burdock extract different concentrations. Plus (+) sign represents bacteria growth and minus (-) sign represents no growth.

Bacteria	aqueous-methanol burdock extract different concentrations (mg/L)										
	control	0.02	0.04	0.08	0.16	0.63	1.25	0.31	2.50	5	10
L.garvieae	+	+	+	+	+	+	+	+	+	+	-
A.hydrophila	+	+	+	+	+	+	+	+	-	-	-
Y. ruckeri	+	+	+	+	+	+	-	-	-	-	-
P. putida	+	+	+	+	+	+	+	+	-	-	-

Table 4: Determining minimum bactericidal concentration (MBC) of aqueous-methanol burdock extracts in different concentrations.

Bacteria	aqueous-methanol burdock extract different concentrations (mg/L)										
	control	0.02	0.04	0.08	0.16	0.63	1.25	0.31	2.50	5	10
L.garvieae	+	+	+	+	+	+	+	+	+	+	+
A.hydrophila	+	+	+	+	+	+	+	+	+	+	-
Y. ruckeri	+	+	+	+	+	+	+	+	+	-	-
P. putida	+	+	+	+	+	+	+	+	+	+	-

Growth has been indicated as plus symbol (+) and no growth has been represented as minus symbol (-).

Determining antibacterial activity of burdock aqueous-methanol extract by agar diffusion assay

The results of investigating antibacterial activity of burdock aqueous-methanol extract and standard antibiotic (tetracycline) by disk-diffusion agar method have been shown in Table 5. According to the results, aqueous-methanol extract had inhibitory effects on *Y. ruckeri* in all concentrations (5, 10, 15, 20, 25 mg/mL). As aqueous-methanol extract concentration increased, the diameter of the bacterial growth inhibition halo also increased significantly and there was a significant difference among all the pathogens under survey except *P. putida* and *A. hydrophila* in 400 and 600 mg/mL concentrations (p>0.05).

Table 5: The diameter of the bacterial growth inhibition (in mm) in the presence of different concentrations of aqueous-methanol burdock extracts.

Bacteria	Tetracycline antibiotic (30µg / Disk)	aqueous-methanol burdock extract different concentrations (mg/mL)				
		50	100	200	400	600
Y. ruckeri	10.45±3.34	8.10±0.57	9.8±0.28	11.6±0.52	13.5±0.50^{a}	16.80±0.50^{a}
L.garvieae	14.38± 0.94	-	-	-	-	7.3±0.57^{c}
P. putida	12.75±0.87	-	-	-	8.3±0.37^{b}	9.9±0.87^{b}
A.hydrophila	18.15±0.45	-	-	-	8±0.67^{b}	9.8±0.49^{b}

Similar letters in each row indicating no significant difference in different concentrations (p>0.05).

Discussion

The increased incidence of bacteria resistance to many antibacterial drugs is of great concern and medicinal plants have proven as an alternative source of antibacterial agents.A recent study evaluated the antibacterial activity of a phytotherapeutic agent prepared from an ethyl acetate fraction (AcOEt) extracted from *Arctium lappa*. This agent inhibited the growth of all tested microorganisms (*Pseudomonasaeruginosa*, *Escherichia coli*, *Lactobacillus acidophilus*, *Streptococcus mutans* and *Candida albicans*) (Popescu *et al*., 2010). Solvent type significantly influences total phenolic content (p<0.05). The highest phenolic content was reported in aqueous-methanol extract. Overall, the best results were obtained from water-methanol mixture (417±0.9 mg GA g^{-1} DW), while the lowest content was found in water extract (Fig. 1). Phenol compounds contain one or more hydroxyl groups (polar part) attached to an aromatic ring. Due to its polarity, this conformation distinct phenols from

other molecules and phenols solubility in solvents can be explained by this conformation (the coexistence of polar and non-polar parts in a molecule) and intermolecular forces between the molecules and solvent as well (Galanakis *et al*., 2013). Water as a solvent creates a hyper polar environment, appropriate for isolation of bioactive compounds with higher polarity, whilst water-methanol, water-ethanol, ethanol and methanol solvents are convenient to extract the molecules with a broad spectrum of polarities due to the presence of the environment created by adding water to these organic solvents (Sun *et al*., 2015). On the other hand, water is necessary for cell swelling and hence in the presence of adequate volume of water in organic solvent, cell wall permeability enhances and can easily break down. Nonetheless, in absolute ethanol or methanol (100%) there is no water available for swelling, so the mixture of two solvents (the mixture of 50% (v/v) water with methanol or methanol) is more optimal and effective (Charpe and Rathod, 2014). According to the reports, due to the higher polarity, the mixture of water and ethanol or methanol are more effective to extract phenolic compounds compared with their corresponding absolute alcohols (Sultana *et al*., 2009). In the current study, in spite of water high polarity compared with water-ethanol, water-methanol, methanol and ethanol solvents, this inorganic solvent displayed a low efficiency to extract phenolic compounds. The mechanism underlying this lesser efficiency may be explained by the water potential to dissolve proteins, polysaccharides and other polar compounds. As another reason, due to the semi-polar nature of phenolic compounds present in burdock root, these compounds have dissolved in water with less efficiency. In a research performed by Ghasemzadeh *et al*.(2015) on rice bran extract, the highest amount of total phenolic content was reported 288.40 mg gallic acid in 100 g of dry matter. The extraction was performed by ultrasound using water-ethanol mixture as solvent (50:50) and was compared with the extract dissolved in ethanol. Furthermore, Nazir *et al*. (2013) conducted a study on phenolic compounds content dissolved in different solvents in persimmon and in consistent with our results, demonstrated that phenolic content was higher in water-ethanol mixture compared with ethanol or water solvents, implying that polarity and viscosity are two properties involved in releasing phenolic compounds from cells (Turkmen *et al*., 2006; Hayouni *et al*., 2007).

DPPH radical scavenging activity testing is an important technique to evaluate extract potential to donate hydrogen or inhibit free radicals. DPPH is a stable free radical which shows a maximum absorption at 517 nm. Due to the reaction between DPPH with antioxidants or hydrogen donor compounds, absorption is reduced which consequently causes a color change from purple to yellow (Ansari *et al*., 2013). Our results evinced that the

maximum calculated IC50 for different extracts differ significantly (p<0.05). The lowest IC50 value was 0.955 mg/mL for aqueous-methanolic extract (50:50 v/v), implying on the higher antioxidant capacity of this extract and aqueous extract showed the lowest free radical scavenging activity. Ling *etal*. (2009) accomplished a research and demonstrated that mango leaves extract dissolved in ethanol solvent and water displayed the highest and lowest free radical scavenging and antioxidant activity, respectively. Rezaeizadeh *et al*. (2011) investigated antioxidant activity of methanol and chloroform extracts of *Momoridica charanita* plant and claimed that the higher free radical scavenging activity of methanol extract (compared with chloroform extract) is due to the presence of higher phenolic content in this solvent and also there are a linear relationship between phenolic content and DPPH radical scavenging activity which is consistent with our findings. Radical scavenging activity of phenolic compounds can be attributed to their hydroxyl groups and enhances by increasing the concentration of phenolic compounds and increased number of hydroxyl groups and thus the probability of donating hydrogen to DPPH free radical, giving rise to the elevation of extract antioxidant and radical scavenging activity.

According to the correlation results in Table 1, phenolic compounds account for 97% of DPPH antioxidant and radical scavenging activity. This correlation has been proven by Ramli and colleagues in 2014, as well. Anokwuru *et al*. (2015) conducted a study on the association between phenolic compounds and antioxidant activity of Terminalia sericea burch plant and reported that there was a negative correlation between phenolic content and IC50 of DPPH radical scavenging activity (r=0.209) which the findings are consistent with our results. Therefore, it can be concluded that due to the presence of a large quantity of phenolic compounds, burdock root extract is associated with increased antioxidant potential.

We examined the effects of different solvents on phenolic compounds content of burdock root extract by HPLC and identified the compounds namely trans-ferulic acid, hesperidin, ferulic acid, vanilin, rosmarinic acid, quercetin, hesperetin and chloregenic acid in the root extract. In our results, methanol was recognized as the best solvent to extract trans-ferulic acid and vanilin. The highest rosmarinic acid content was found in water-methanol solvent and 100% ethanol contained the highest quantity of chloregenic acid. Water had the maximum extraction yield of quercetin, hesperidin and hesperetin. Nevertheless, collectively water-methanol mixture has exhibited a higher yield for isolation of phenolic compounds and this may be due to the semi-polar nature or the less polarity of phenolic compounds in burdock root extract which make them less soluble in water, causing to decreased yield. Kallithraka *et al*.(1995) investigated the effects of different solvents on the phenolic compound extraction yield

from grape seeds and identified gallic acid compounds, catechin, epicatechin, epicatechin gallate and procyanidin dimers and trimmers using HPLC approach. According to their findings, methanol yielded the greatest quantity of catechin (+), epicatechin(-) and epicatechin gallate. Additionally, acetone and 75% ethanol extracted the highest amount of proanthocyanidins and gallic acid, respectively. Overall, 75% acetone has yielded higher phenolic compounds quantity **Pekić** *et al.*(1998) conducted a study on grape seed extracts dissolved in water-acetone and water-ethyl acetate solvents and revealed that proanthocyanidins is not able to be efficiently extracted in the absence of water and increasing water content will remarkably enhance yield. However, the excessive increase in water content causes a less selective extraction. Overall results indicated that proanthocyanidins can be selectively extracted by ethyl acetate containing 10% water.

Due to higher efficiency of water-methanol mixture to isolate active ingredients, this solvent was employed to investigate antimicrobial activity of burdock root extract. Alizadeh Behbahani *et al*. (2013) examined antimicrobial activity of *Avicennamarina* leaves extracts dissolved in water, ethanol, methanol or glycerine against *Penicilliumdigitatum* and reported that due to the higher efficiency of ethanol and methanol solvents to isolate active ingredients and subsequent increased dry mass, methanol or ethanol leaves extracts exert greater inhibitory and fungicidal effects against *Penicillium digitatum*. Our study demonstrated that the higher the concentration of water-methanol burdock root extract, the larger the diameter of the bacterial growth inhibition halo of *Y. ruckeri* and in all concentrations the diameter of halos were significantly different with tetracycline halo. There was a significant difference among all bacterial strains treated with 400 and 600 (mg/mL) concentrations of burdock root extract except *P. putida* and *A. hydrophila* (Table 2). Sagdic *et al*. (2002) conducted a study to investigate antibacterial capacity of sage, thyme, oregano and cumin essences against *E. coli* and in consistent with our results they found that, as the concentration of the extracts increase, the diameter of inhibition also increases. Depending upon the type of organism, antimicrobial activity of burdock water-methanol extract varied, so that *Y. ruckeri* as a gram negative bacterium was found to be the most susceptible one, compared with other pathogens (Table 2) while *L. garvieae* as a gram positive bacterium was recognized as the most resistant strain. Moreover, comparing the results of disk diffusion testing by using tetracycline as control evinced that water-methanol extract of burdock root extract displayed a relatively higher antibacterial activity against *Y. ruckeri*, compared with control group. Hitherto, few studies have been performed focusing on antibacterial activity of medicinal herbs against *Y. ruckeri.* Alishahi *et al*.(2012)

showed that pomegranate peel, *Nigella Sativa* and *Zataria multiflora* extract had appropriate antibacterial effects against this strain, so that the diameter of growth inhibition halo was 22, 20 and 16 mm, respectively. Habibipour and Rajabi (2015) accomplished an in vitro survey to evaluate antimicrobial activity of burdock extract and *Artemesia absinthium* extract and found that burdock extract had antibacterial effects on *Haemophilus influenza* and *P. aeraginosa* and the diameter of bacterial growth inhibition halo was 18.4 and 11.9 (in mm), respectively. This activity can be clarified by the presence of lignan compounds including arctigenin and arctin which are among the major components in burdock root extract. These secondary metabolites function as anti-cancer, anti-inflammtion and antibacterial agents exert their effects by targeting cell membrane (Kamkaen *et al*., 2006).

The results of Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) testing of burdock root methanol-water extract indicated that *L. garvieae* was the most resistant pathogen (MIC, 10 mg/mL), while MIC values of burdock root methanol-water extract for *Y. ruckeri*, *P. putida* and *A. hydrophila* were 1.25, 2.5 and 2.5(mg/mL), respectively. MBC of burdock root methanol-water extract against *Y. ruckeri*, *P. putida* and *A. hydrophila* was obtained 5, 10 and 10 mg/mL, respectively. Habibipour and Rajabi (2015) reported that MIC and MBC of burdock root methanol-water extract against *H. influenza* was 230 and 540 mg/mL, respectively. However, these values were 45 and 90 mg/mL for *A.absinthium*. MIC and MBC values of burdock root methanol-water extract against *P. aeruginosa* were 500 and 750 mg/mL and of *A. absinthium* were 285 and 430 mg/mL. In both of them, MIC value against *Bacillus cereus* was 166 mg/mL. Antimicrobial properties of burdock plant can be explained by the presence of lignan and phenolic compounds, giving rise to enhancing permeability and, bacterial cell wall breakdown and subsequently extracellular discharge of cellular content (Kamkaen *et al.*, 2006).

The results of this study indicated that burdock root extract contains a substantial quantity of phenolic compounds with antioxidant capacity which can be used as natural antioxidant source. According to our findings, compared with other solvents, water-methanol solvent is more efficient to isolate phenolic compounds and antioxidants and antioxidant capacity enhances, as the quantity of phenolic compound in extract increases. Therefore, due to the higher efficiency to isolate active ingredients from burdock root extract, methanol-water solvent was employed to examine antimicrobial activity of burdock root extract. Among all the pathogens tested, *Y. ruckeri* was recognized as the most sensitive strain. However conducting extensive studies in clinical settings in fish farms seems necessary. We believe that after determining effective concentrations, understanding the

underlying mechanisms and proving its safety, this extract can be introduced as a promising efficient natural antimicrobial agent to aquaculture industry as an alternative to chemical drugs and antibiotics to treat yersiniosis.

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